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Modulation of Sulfur Mustard Toxicity by Arginine Analogues and Related Nitric Oxide Synthase Inhibitors *in Vitro*

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The modulating effects of a series of arginine analogues and related nitric oxide synthase inhibitors against the toxicity of sulfur mustard (HD) in primary cultures of chick embryo forebrain neurons were examined. In addition to the previously identified protective compounds, D- and L-nitroarginine methyl ester, eight additional arginine analogues were shown to have significant, concentration-dependent protective characteristics against HD toxicity. Of these, L-nitroarginine was the most potent, increasing the LC50 of vehicle-pretreated HD-treated control cultures by ~350%. In addition to these protective agents, five compounds related to arginine were also identified that potentiated the toxicity of HD in the neuron cultures in a concentration-dependent manner. This action occurred at concentrations where these chemicals alone exhibited no toxicity. Characterization of the active compounds in this study showed that it was likely that the protective agents, as well as those compounds that potentiated HD toxicity, were exerting their effects at the same biochemical target, but not through the inhibition of nitric oxide synthase. Although the identity of this target site is as yet unknown, these studies demonstrate that subtle alterations to the arginine structure can yield compounds that differentially modulate the toxicity of HD through their activity at a common target site. © 1998 Society of Toxicology.

Sulfur mustard (bis (2-chloroethyl) sulfide, NATO Standard Agreement designation; HD) is a vesicating, or blister-causing, agent that has found widespread use in chemical warfare (CW). From its first use in 1917 at Ypres, Belgium, to its most recently documented use during the Iran–Iraq war (United Nations, 1986, 1987, 1988), this compound has been the subject of intensive investigation to ascertain its mechanism of toxic action. These studies have been largely unsuccessful, partially due to the lack of good animal models of vesication, but more importantly due to the extreme chemical reactivity of HD. Not only does HD extensively alkylate DNA, but it also reacts with an enormous variety of different low-molecular-weight compounds that are routinely present in cells and tissues (Papirmeister *et al.*, 1991). Presumably, this reactivity at critical target sites leads to the multiplicity of toxic effects that HD exerts, including vesication, mutagenicity, carcinogenicity, and toxicity, to a variety of cell types and organ systems (National Academy Press, 1993; Papirmeister *et al.*, 1991; Smith and Dunn, 1991; Willems, 1989). Until recently, no effective antidotes to these effects either *in vitro* or *in vivo* have been identified.

Nitric oxide (NO) is a biomolecule that has received a tremendous amount of research attention during the last several years due to recent revelations that it is responsible for, or is involved in, a great many roles that are vital for normal bodily physiology and pharmacology (Bredt and Snyder, 1992, 1994; Culotta and Koshland, 1992; Koshland, 1992; Kerwin and Heller, 1994; Nathan and Hibbs, 1991; Nussler and Billiar, 1993). We noted that disturbances of many of these NO-mediated functions would result in cellular toxicities that were reminiscent of HD-induced toxicities, including DNA damage, cytotoxicity, inflammation, and effects on the immune, cardiovascular, and central nervous systems. It therefore seemed reasonable to hypothesize that the toxicity of HD may be the result of HD-induced overproduction of toxic quantities of NO. Accordingly, inhibitors of nitric oxide synthase (NOS) should therefore prevent the toxicity of HD. This approach was successful and the well-characterized NOS inhibitor L-nitroarginine methyl ester (L-NAME) was found to confer a degree of protection to cultured neurons that was in excess of those obtained by any previous drug regimens against the toxicity of HD *in vitro* (Sawyer *et al.*, 1996). Surprisingly, however, the protective characteristics of L-NAME were shown to be not associated with its NOS inhibiting ability, and by extension, NO overproduction seemed to have little to do with HD cytotoxicity.

We have further characterized the protective effects of L-NAME against the toxicity of HD (Sawyer, 1998) and found that not only does it confer protection against HD when used as a pretreatment, but also when used up to several hours after HD treatment. However, we have not thus far been successful in identifying the mechanism of its protective action. L-NAME is only one of several arginine analogue competitive inhibitors of NOS, and these represent only one of several classes of NOS inhibitors. It seems reasonable that if L-NAME is inhibitory toward both NOS and HD toxicity, then the active site of NOS and the target(s) of HD toxicity may be similar enough so that other

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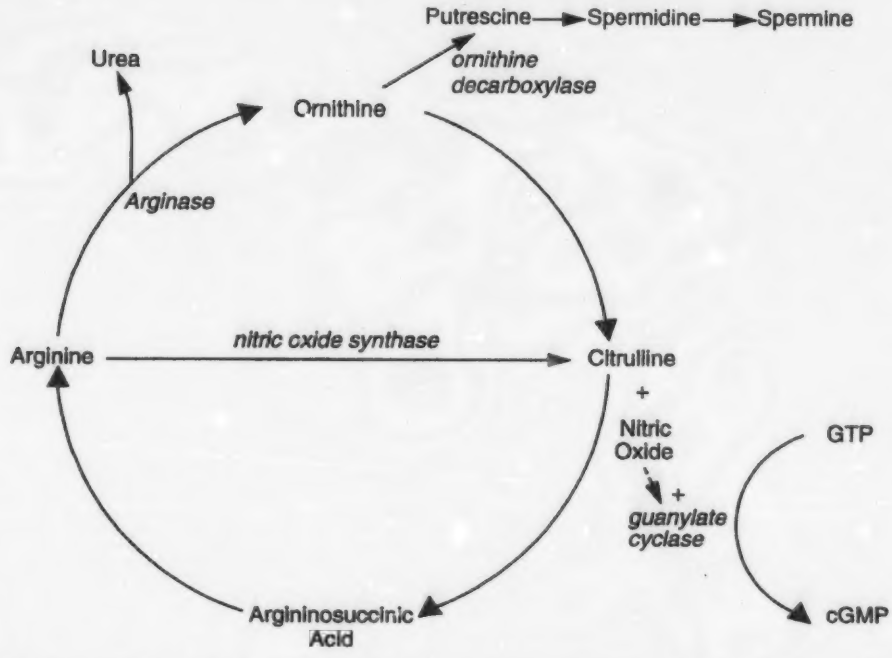

FIG. 1. Biochemical pathways utilizing arginine. Note that nitric oxide activates guanylate cyclase and promotes the conversion of GTP to cGMP.

classes of NOS inhibitors will also modulate the toxicity of HD. In this report we describe the results of studies that investigate the modulation of HD toxicity by a variety of arginine analogue NOS inhibitors and arginine-related compounds that are involved in other metabolic pathways (Fig. 1) in order to further understand the mechanism of HD toxicity and L-NAME-mediated protection, as well as to identify additional compounds that are protective against HD toxicity.

MATERIALS AND METHODS

Chemicals. Penicillin–streptomycin, fungizone, and trypsin were purchased from Flow Laboratories (Mississauga, Ontario, Canada) and a modified minimum essential medium (mMEM) (Hertz *et al.*, 1982) was prepared. 2-Amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT), L-N^6-(1-iminoethyl)-lysine hydrochloride (L-NIL), L-N^5-(1-iminoethyl)ornithine dihydrochloride (L-NIO), N^G,N^G-dimethyl-L-arginine dihydrochloride (ADMA), S-methylisothiourea sulfate (ITU), diphenylene iodonium chloride (DPIC), (+)-S-2-amino-5-iodoacetamidopentanoic acid (AIAP), (+)-S-2-amino-6-iodoacetamidohexanoic acid (AIAH), N^G-monomethyl-L-arginine (L-NMMA), and 7-nitroindazole (7-NOI) were purchased from Alexis Corporation (San Diego, CA). L-Canavanine, aminoguanidine (AG), L-citrulline, N^G-nitro-L-arginine (L-NOARG), N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), N^G-nitro-D-arginine methyl ester hydrochloride (D-NAME), L-lysine, phospho-L-arginine (P-ARG), L-argininic acid, L-homoarginine, L-ornithine, L-arginine and D-arginine, N^G-nitro-L-arginine benzyl ester (L-NABE), methylguanidine (MeG), guanidine, spermidine, and spermine were acquired from Sigma Chemical Company (St. Louis, MO). L-Arginine methyl ester (L-AME) and argininosuccinic acid were obtained from ICN Biomedicals (Montreal, Quebec, Canada). Methylene blue and N^G-nitro-D-arginine (D-NOARG) were purchased from Research Biochemicals International (Natick, MA). The structures of the test compounds used in this study are shown in Fig. 2. AlamarBlue was acquired through AccuMed International, Inc. (Westlake, OH). Sulfur mustard was prepared by the Chemical Biological Defence Section at greater than 99% purity.

Neuron culture. Primary cultures of chick embryo neurons were initiated from 8-day-old embryos as previously described (Weiss and Sawyer, 1993). Cultures were seeded at a density of 100,000 cells/well in 96-well titer plates in mMEM supplemented with streptomycin (10 μg/mL), penicillin (10 units/mL), fungizone (0.25 μg/mL), and 5% horse serum. HD treatments were routinely carried out on day 8 and cytotoxicity assessments were carried out 24 h later. Cell viability was assessed using alamarBlue, a nontoxic indicator dye which is reduced by viable cells to a water-soluble species whose absorbence (600–570 nm) can be measured in the medium.

Chemical treatment and inhibitor studies. On the day of chemical treatment the cultures were treated with freshly prepared treatment medium so that the desired final HD concentration was reached at 0.25% ethanol (v/v). Stock solutions of test inhibitors were freshly prepared in complete medium just prior to use, usually 1 h prior to HD treatment. To assess cytotoxicity, after the cells had incubated with HD for 19 h, alamarBlue was added (10%, v/v) and the cultures were allowed to incubate an additional 5 h. The absorbences (570–600 nm) were then read on a Thermomax titer plate reader (Molecular Devices, Sunnyvale, CA). Median lethal concentration (LC50) values were determined graphically and the percent protection against HD was expressed as the LC50 of drug-treated, HD-exposed cultures divided by the LC50 of vehicle-treated, HD-exposed cultures × 100.

Several compounds which exacerbated HD toxicity in these experiments were selected for further study, including L-canavanine, AG, and L-AME. These drugs were examined for their effects on HD toxicity in immature cultures by treating cells on day 1 of culture (day 0 is designated as the day of seeding) and assaying for viability 24 h later. In addition, these compounds were also assayed for their effects on HD toxicity when administered after HD culture treatment. In these experiments, the cultures were

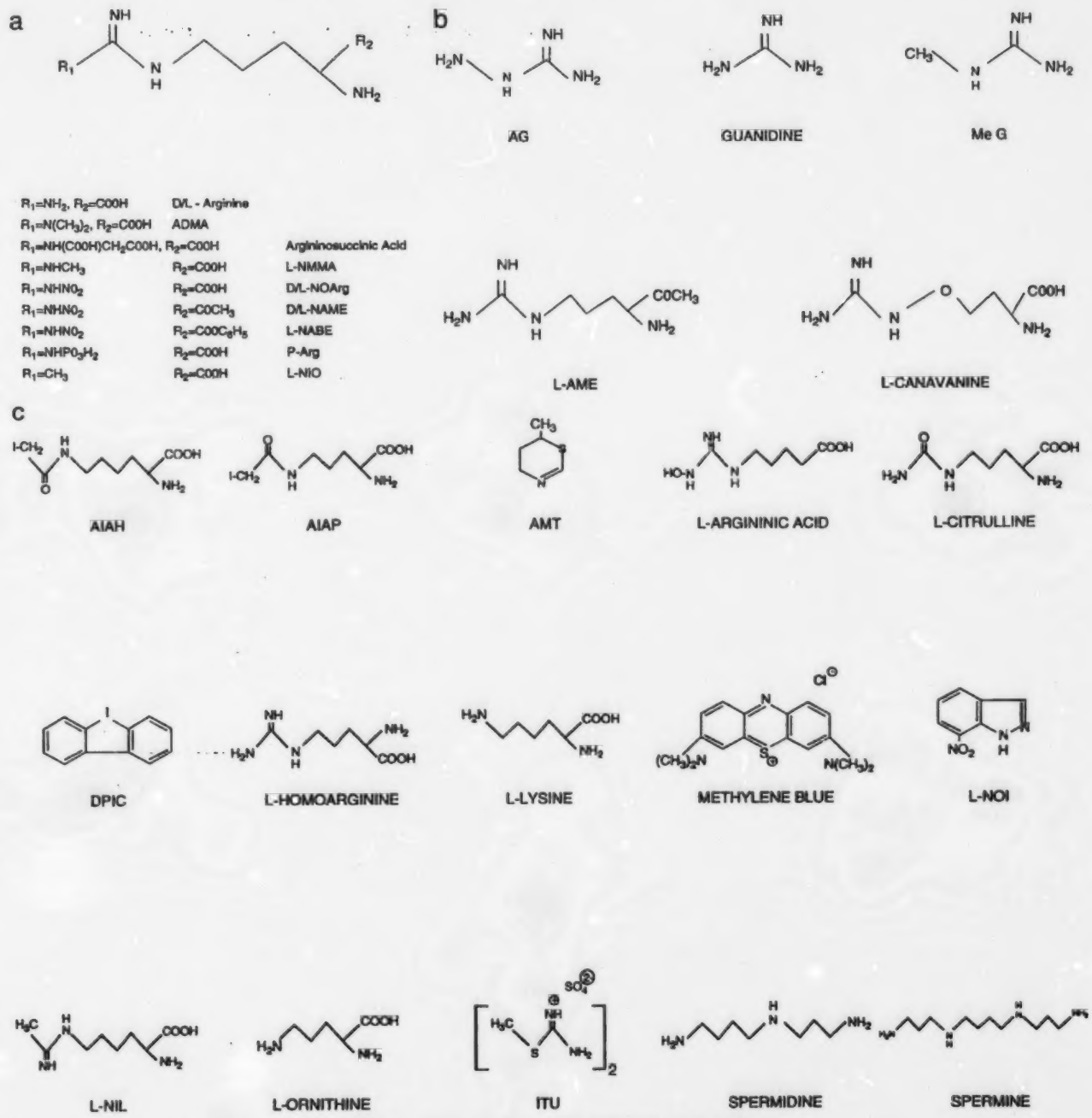

FIG. 2. Structures of test compounds used in this study.

treated for 2 h with HD. The treatment medium was then removed and replaced with medium previously "conditioned" by cultures of a similar age. The cultures were then treated with the treatment drug. Finally, the effects of combining the protective drug L-NAME with compounds which potentiated HD toxicity were examined. In these experiments, the cultures were treated simultaneously with L-NAME and one of the drugs which increased HD toxicity. The results were then compared to the effects of individual drug treatment on HD toxicity.

Statistical methods. Data were analyzed using one-way analysis of variance. Subsequent to this, test groups were compared with vehicle-treated control cultures using the Dunnett's test (Tallarida and Murray, 1990). Significant differences from control were assumed if p values were less then 0.05.

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TABLE 1a
Effect of Arginine Analogues and Related NOS Inhibitors on the Toxicity of HD in Neuron Culture

	Pretreatment concentration (%)						
Pretreatment	Control	0.1 mM	1.0 mM	2.5 mM	5.0 mM	10.0 mM	15 mM
ADMA	100	108.5 ± 12.4	108.7 ± 13.8	112.9 ± 10.5	119.6 ± 23.2	115.1 ± 21.2	143.9 ± 25.9
Guanidine	100	98.2 ± 0.6	80.0* ± 7.2	68.9* ± 1.6	—	—	—
MeG	100	102.3 ± 1.2	86.2* ± 4.1	73.7* ± 3.9	67.9* ± 7.6	—	—
L-NABE	100	112.0 ± 15.9	136.4 ± 14.7	112.3 ± 22.6	—	—	—
D-NAME	100	100.4 ± 3.6	168.2 ± 42.4	236.1* ± 36.8	284.9* ± 10.2	276.5* ± 3.8	—
L-NAME	100	107.1 ± 6.9	178.6 ± 43.3	229.4* ± 41.5	246.8* ± 35.1	240.5* ± 10.5	—
L-NIO	100	101.7 ± 18.7	107.4 ± 13.3	102.0 ± 9.4	105.2 ± 17.1	84.6 ± 10.9	86.5 ± 34.4
L-NMMA	100	90.8 ± 4.6	100.5 ± 6.5	117.0 ± 14.9	112.9 ± 9.6	121.4 ± 3.9	130.6* ± 6.3

RESULTS

Tables 1a–1c show the results obtained when screening a series of arginine analogue NOS inhibitors and related compounds for their efficacies as protective agents against the toxicity of HD. In these studies the upper concentration limits tested were determined by solubility, toxicity, the upper limits of the concentration–response, or economics; many of the inhibitors tested in this study have become available only recently and their costs usually reflected this. No significant modulation of HD toxicity was observed by ADMA, AIAH, AMT, L-argininic acid, argininosuccinic acid, ITU, L-homoarginine, L-lysine, methylene blue, L-NABE, L-NIO, L-ornithine, L-spermidine, and L-spermine. Significant protection was obtained at high concentrations of L-arginine, D-arginine, L-citrulline, and L-NMMA. In comparison, D-NAME and L-NAME showed similar protective capacities to each other, with protection evident at 1.0 mM and maximal by 5.0 mM. This protection was similar whether the cultures were treated 1 h prior to or 2 h after HD exposure (data not shown). In addition, these two compounds were not protective against HD toxicity in immature cultures (data not shown). Several compounds were found to exacerbate the toxicity of HD. These included AIAP, DPIC, guanidine, MeG, and 7-NOI.

Figure 3 shows a typical concentration–response of mature neuron cultures exposed to HD, as well as the protection afforded against this toxicity by the most effective of the compounds tested in this study, L-NOARG. Protection was evident by 1.0 mM L-NOARG and maximal by 12.5 mM L-NOARG, increasing the LC50 of HD by ~350%. This is also illustrated by Fig. 4, which depicts the protection in terms of LC50 multiples and also shows the protective effects of D-NOARG, L-NIL, and P-ARG. All three compounds yielded significant concentration-related protective effects which peaked at ~200% (D-NOARG), ~170% (P-ARG), and ~190% (L-NIL).

Figure 5 illustrates the modulating activities of L-canavanine, AG, and L-AME on HD toxicity. In every case these compounds increased HD toxicity in a concentration-dependent manner, increasing the toxicity of HD by ~250% (L-AME) to ~400% (L-AG). This effect occurred at concentrations at which the compounds were not toxic when administered alone. These compounds potentiated the toxicity of HD whether they were used as a pretreatment or 2 h post-HD treatment (Fig. 6) and did not have any effect on the toxicity of HD in immature neurons (Fig. 7). Figure 8 depicts the additive effects of L-canavanine, AG, or L-AME with L-NAME on HD toxicity. When L-canavanine and AG were tested, the (additive) calculated LC50 value of combining these compounds with the

TABLE 1b
Effects of Arginine Analogues and Related NOS Inhibitors on the Toxicity of Sulfur Mustard in Neuron Culture

	Pretreatment concentration (%)						
Pretreatment	Control	5.0 mM	10 mM	15 mM	25 mM	50 mM	100 mM
D-Arginine	100	104.0 ± 11.8	119.4 ± 25.0	—	145.9* ± 17.5	146.4* ± 5.7	—
L-Arginine	100	112.9 ± 13.0	122.5 ± 14.8	—	142.0* ± 19.9	151.9* ± 15.2	—
L-Argininic acid	100	107.6 ± 10.4	104.4 ± 8.0	125.7 ± 25.8	130.3 ± 11.8	—	—
L-Citrulline	100	104.3 ± 25.5	117.2 ± 20.2	123.4 ± 9.6	127.7 ± 17.5	129.7 ± 26.0	152.7 ± 17.7*
L-Homoarginine	100	93.7 ± 10.7	93.3 ± 8.2	100.9 ± 10.6	99.5 ± 15.5	—	—
L-Lysine	100	111.8 ± 14.7	112.5 ± 8.4	—	120.9 ± 5.5	117.3 ± 9.8	—
L-Ornithine	100	110.2 ± 6.2	102.4 ± 20.3	97.7 ± 22.3	96.1 ± 10.9	107.3 ± 9.8	—

TABLE 1c
Effects of Arginine Analogues and Related NOS Inhibitors on the Toxicity of HD in Neuron Culture

Pretreatment	Pretreatment concentration (%)						
	Control	0.01 μM	0.1 μM	1.0 μM	10.0 μM	100 μM	1.0 mM
AIAH	100	—	—	—	95.9 ± 4.8	—	—
AIAP	100	—	—	—	74.2 ± 9.0*	—	—
AMT	100	—	—	—	98.7 ± 4.1	93.2 ± 6.4	—
Argininosuccinic acid	100	—	—	104.3 ± 11.6	101.2 ± 7.5	103.2 ± 8.5	—
DPIC	100	113.1 ± 11.4	73.3* ± 14.4	—	—	—	—
ITU	100	—	—	—	—	106.9 ± 12.4	91.7 ± 13.9
Methylene blue[a]	100	110.5 ± 10.6	127.2 ± 19.5	—	—	—	—
L-NOI	100	—	—	—	87.7 ± 9.4	70.8 ± 13.1	—
Spermidine	100	—	—	—	—	96.2 ± 5.1	—
Spermine	100	—	—	—	—	107.4 ± 29.9	—

Note. Test compounds were tested for their modulatory effects on the toxicity of HD in 8-day-old cultures. Cytotoxicity was assessed at 24 h post-HD exposure and protection was expressed as a percentage of the LC50 obtained from HD-treated cultures not receiving drug pretreatment. Results represent the mean ± SD of at least three separate experiments (except D/L-NAME, $n = 2$). The values denoted by an asterisk are significantly different from vehicle-pretreated controls (Dunnett's test, $p < 0.05$).

[a] Data taken from Sawyer *et al.* (1998).

protective L-NAME was similar to the experimental values obtained. However, the combination of L-AME and L-NAME yielded an LC50 that was significantly higher than the calculated value.

DISCUSSION

The mechanism of action of HD has long been the subject of investigation. Despite intensive efforts, however, how HD exerts its toxic effects is still unknown and effective antidotes against this CW agent do not exist. In recent work (Sawyer *et al.*, 1996), we hypothesized that HD-induced overproduction of NO was the cause of the resultant toxicity. Accordingly, inhibitors of NOS should provide some degree of protection against the toxicity of HD. Although the arginine analogue NOS inhibitor L-NAME was shown to confer significant protection against the toxicity of HD in neuronal cell cultures, HD toxicity and L-NAME protection were shown not to be associated with NO (Sawyer *et al.*, 1996). The present study was undertaken to assess whether other arginine NOS inhibitors and related compounds also possess protective activity against HD.

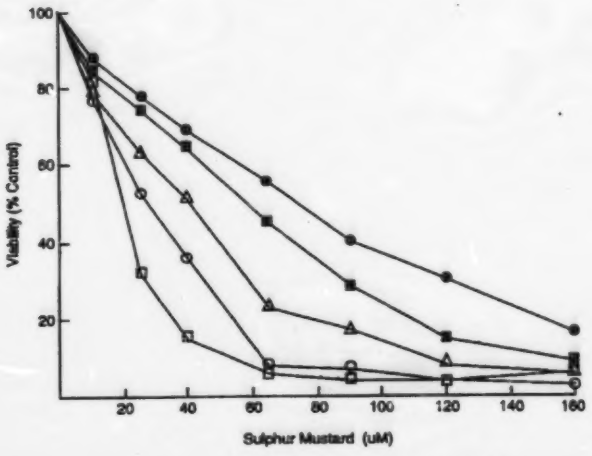

FIG. 3. Concentration-response of HD toxicity and L-NOARG protection in neuron culture. Eight-day-old cultures were treated for 1 h with solvent vehicle (medium, □), and 1.0 mM (○), 2.5 mM (△), 5.0 mM (■), and 12.5 mM (●) L-NOARG prior to HD exposure. Viability was assayed 24 h after HD exposure. Data shown represent a typical experiment utilizing six culture wells per data point.

Baseline data were acquired by once again testing both D-NAME and L-NAME for their efficacy as protective drugs against HD toxicity. As in previous studies (Sawyer, 1998; Sawyer *et al.*, 1996), both compounds provided 200–250% protection against HD in terms of increasing the LC50 compared to solvent (medium)-pretreated, HD-treated control cultures. These two compounds are more water-soluble analogues of the potent NOS inhibitor L-NOARG (Moore *et al.*, 1990) and not surprisingly, both D-NOARG and L-NOARG also conferred excellent protection against HD, with L-NOARG increasing the LC50 of HD-treated cultures by ~350%. Interestingly, although D- and L-NAME were equipotent, D-NOARG was significantly less protective (LC50 ~200%) than L-NOARG. The reasons for this anomaly are unclear; however, it is possible that esterification of nitroarginine lessens its affinity to a particular site, as well as site stereospecificity. This is also partially supported by the data obtained from experiments using D- and L-arginine, where both stereoisomers gave identical protection, albeit at very high mM concentrations.

Although our findings with the D isomers of arginine, NAME, and NOARG once again ruled out the possibility that

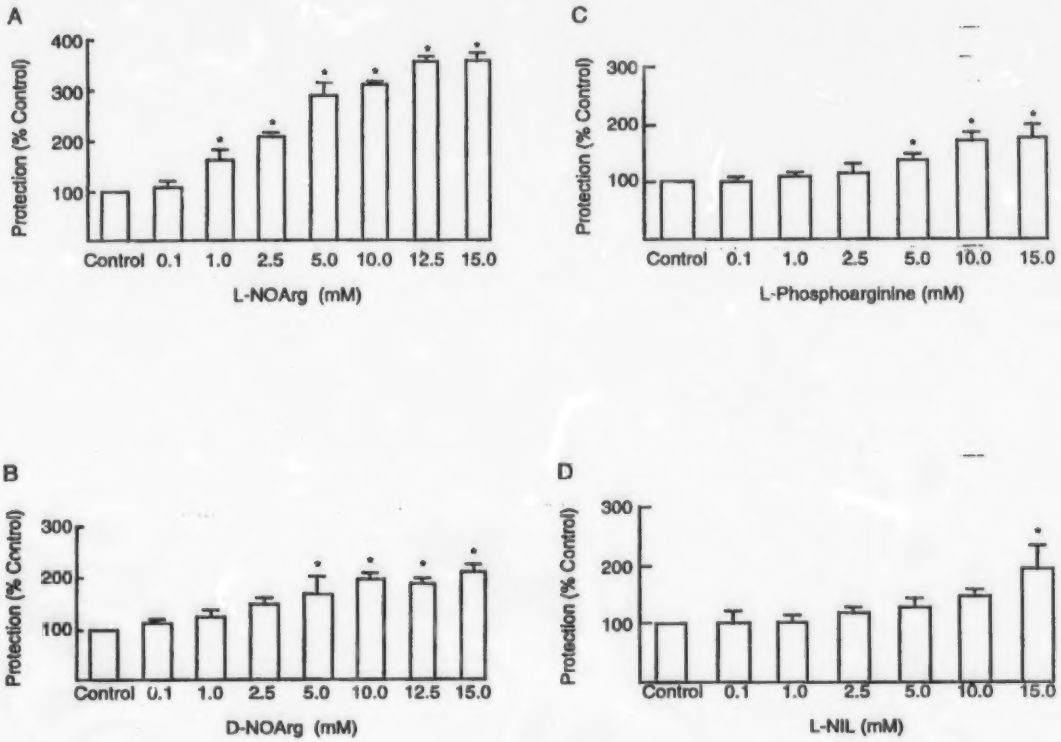

FIG. 4. Protective effects of arginine analogues against HD toxicity in neuron culture. Eight-day-old cultures were treated for 1 h with L-NOARG (A), D-NOARG (B), P-ARG (C), or L-NIL (D) prior to HD exposure. The cultures were assayed for viability 24 h after HD exposure. Values represent the mean ± standard deviation of at least three experiments and are normalized against the LC50 of vehicle pretreated HD exposed cultures. Asterisks denote significant differences from control values (Dunnett's test, $p < 0.05$).

these compounds were exerting their protective effects through NOS inhibition, it was clear that arginine and particularly some of the arginine analogue NOS inhibitors possessed protective effects against HD toxicity. Therefore, additional members of this group were tested. ADMA (Vallance *et al.*, 1992) and L-NMMA (Palmer *et al.*, 1988; Rees *et al.*, 1989; Sakuma *et al.*, 1988) are both potent NOS inhibitors and although ADMA was inactive at the concentrations tested in these studies, L-NMMA had significant protective activity (15 mM, ~130%). A similar situation was also found when two additional NOS inhibitors, L-NIO (Mulligan *et al.*, 1992; Rees *et al.*, 1990) and L-NIL (Moore *et al.*, 1994), were tested. While L-NIO was inactive, L-NIL was relatively active at high concentrations (15 mM, ~190%). Although the number of test compounds and the range of protective values were relatively small, the results were examined with an eye to possibly identifying structure–activity relationships. No clear relationships were apparent, other than the obvious observation that relatively subtle alterations to the arginine backbone can confer protective activity against HD to the molecule.

The first NOS inhibitors to be identified were arginine analogues. However, during the intense research activity that has followed the identification of NO as a potent biomolecule, several additional classes of NOS inhibitors were characterized in the hope that they would exhibit more specificity toward the myriad of different effects for which NOS, and by extension NO, were responsible. Although our experimental results seemed to indicate that the arginine structure was vital with respect to protecting against the toxic effects of HD, it was necessary to investigate the possibility that the active site of NOS and the site where HD exerts its toxicity were similar enough so that other classes of NOS inhibitors would also have protective efficacy against HD. Accordingly, representatives of these other NOS inhibitor classes were tested. The isothioureas are a non-amino-acid arginine analogue class of NOS inhibitor. Only one member of this series, the methyl derivative ITU, was tested. Although a very effective NOS inhibitor (Garvey *et al.*, 1994; Southan *et al.*, 1995; Szabo *et al.*, 1994), ITU caused no modulation of HD toxicity at any of the concentrations that did not by themselves cause toxicity.

The substituted indazoles are among the first classes of compounds that were identified as strong inhibitors of NOS but were not guanidino-substituted derivatives of L-arginine (Babbedge *et al.*, 1993; Medhurst *et al.*, 1994; Moore *et al.*,

1993a,b). One of the most efficacious of these NOS inhibitors, 7-NOI, was tested and found not to protect against the toxicity of HD. Interestingly, however, at the highest nontoxic concentration tested (100 μM), 7-NOI marginally increased the toxicity of HD. The reasons for this effect are unclear, but may have been due to an increase in the sensitivity of the cells to HD at near-toxic concentrations of 7-NOI. AMT is another heterocyclic NOS inhibitor (Nakane *et al.*, 1995) that was tested in these studies and also found not to modulate HD toxicity.

Methylene blue is a well-known biochemical tool whose actions on iron-containing enzymes are generally attributed to

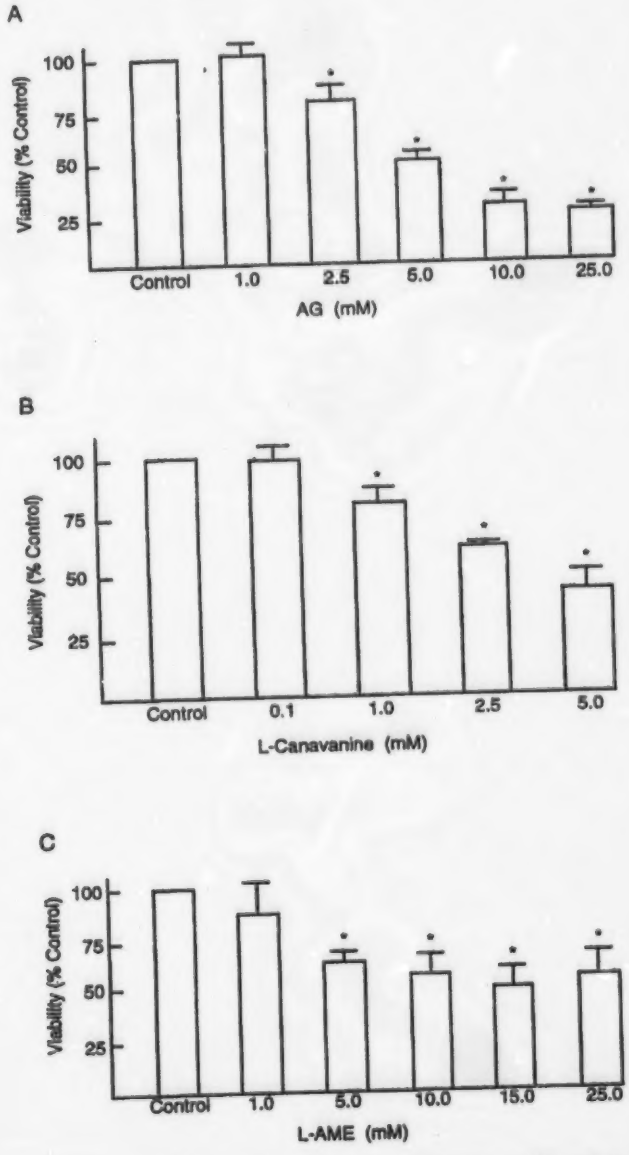

FIG. 5. Modulatory effects of arginine analogues on HD toxicity in neuron culture. Eight-day-old cultures were treated for 1 h with AG (A), L-canavanine (B), or L-AME (C) prior to HD exposure and assayed for viability 24 h later. Values represent the mean ± standard deviation of at least three separate experiments and are normalized against the LC50 of vehicle-pretreated HD-treated control cultures. Astererisks denote significant differences from control values (Dunnett's test, $p < 0.05$).

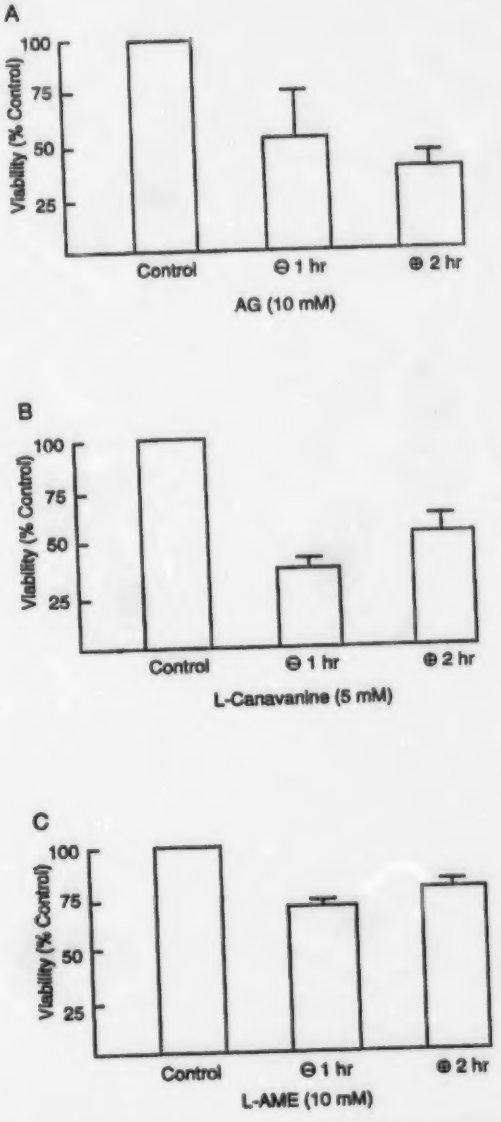

FIG. 6. Effect of pre- or posttreatment of arginine analogues on the toxicity of HD in neuron culture. Eight-day-old cultures were pretreated for 1 h (−1 h) or treated 2 h after HD exposure (+2 h) with AG (A), L-canavanine (B), or L-AME (C) and assayed for viability 24 h later. Values represent the mean ± standard deviation of at least three separate experiments and are normalized against the LC50 of vehicle-pretreated HD-treated control cultures.

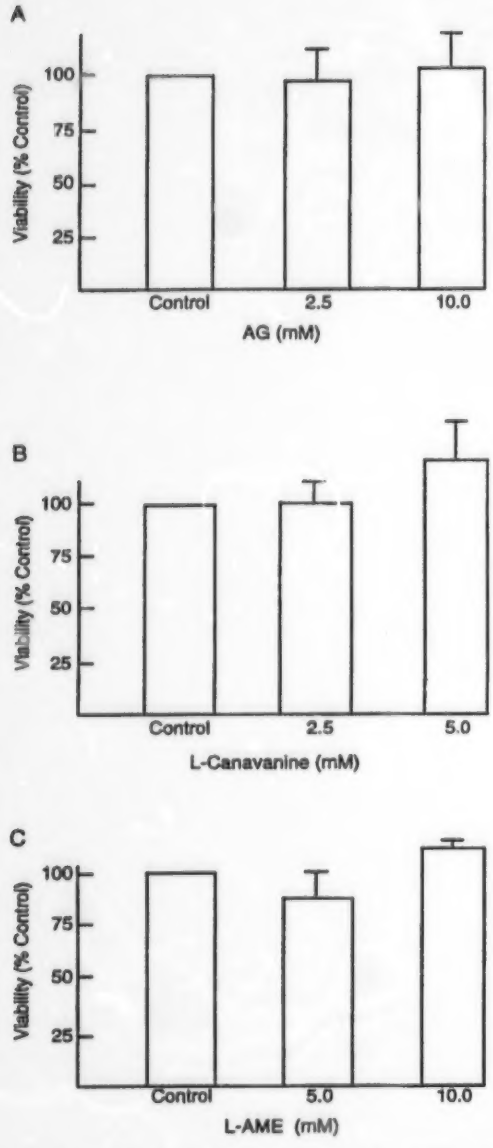

FIG. 7. Effect of arginine analogues on the toxicity of HD in immature neuron culture. One-day-old cultures were treated for 1 h with AG (A), L-canavanine (B), or L-AME (C) prior to HD exposure and assayed for viability 24 h later. Values represent the mean ± standard deviation of at least three separate experiments and are normalized against the LC50 of vehicle-pretreated HD-treated control cultures.

its abili y to oxidize protein-bound heme and nonheme ferrous iron (J iner *et al.*, 1988; Salaris *et al.*, 1991). Its ability to inhibi NO-mediated events is thought to be due primarily to its inhib.don of NO-activated guanylyl cyclase, thus preventing cGMP formation (Fig. 1, Murad, 1994; Ohlstein *et al.*, 1982), although it has also been implicated as a direct NOS inhibitor (Mayer *et al.*, 1993). When tested in neuronal culture, methylene blue was extremely toxic, even at low micromolar concentrations. However, at nontoxic concentrations ($\leq 0.1\ \mu M$), this compound had little effect on the toxicity of HD, indicating that, unlike NO (whose actions also include cytotoxic and tumoricidal activity), HD probably does not exert its toxicity by specifically interacting with iron-containing enzymes.

The effect of DPIC on HD toxicity was also examined in the neuron cultures. This compound is representative of a potent class of NOS inhibitors (McCall *et al.*, 1991; Stuehr *et al.*, 1991) whose effects are due to their inhibitory action on flavoproteins. Similar to methylene blue, DPIC was also extremely toxic to the neuronal cultures. However, at nontoxic concentrations, DPIC marginally increased HD toxicity. This observation may have been due to an increased susceptibility of the cells to HD at near-toxic concentrations of DPIC and indicates that HD toxicity is probably not associated with flavoprotein perturbation.

Following this survey of several of the currently known classes of NOS inhibitors, it was concluded that the structure of arginine, rather than its involvement in the NOS pathway, was the factor that endowed it with the ability to, with subtle alterations, protect against the toxicity of HD. Accordingly, several structurally related compounds were tested for protective activity. Although L-homoarginine, L-argininic acid, L-lysine, and L-NABE were inactive at the concentrations tested, one additional compound, P-ARG, was identified that was quite efficacious in protecting against the toxicity of HD in the neuron cultures. This compound, probably also an NOS inhibitor (no references in the NOS literature were located for this compound), is primarily known as an intermediate energy storage molecule in invertebrates and why it is protective against HD is unknown. However, it does possess structural similarity to another intermediate energy storage molecule in vertebrates, creatine phosphate—a molecule that in preliminary studies, we have also found to possess protective activity against HD in this system. The high-energy phosphate group of creatine phosphate is rapidly transferred to ADP to form ATP by the action of creatine kinase, one of the few enzymes that has been found to be strongly inhibited by HD (Papirmeister *et al.*, 1991). It may be that perturbation of this energy depot system is at least partially involved in HD toxicity. We are currently investigating this possibility.

A final possibility that was necessary to investigate was that HD toxicity was prevented by selected arginine analogues due to their interruption of a (known) biochemical pathway. Different pathways utilizing L-arginine or its metabolites were therefore examined by testing substrates, products, or inhibitors of the pathway. These included biochemicals associated with polyamine metabolism and the urea cycle (Fig. 1). The polyamines, putrescine, spermidine, and spermine, are produced through the initial action of ornithine decarboxylase on ornithine (Fig. 1) and are essential for normal cell growth and differentiation (Morgan, 1987; Pegg, 1988). They have also been suggested as possibly having regulatory activity with

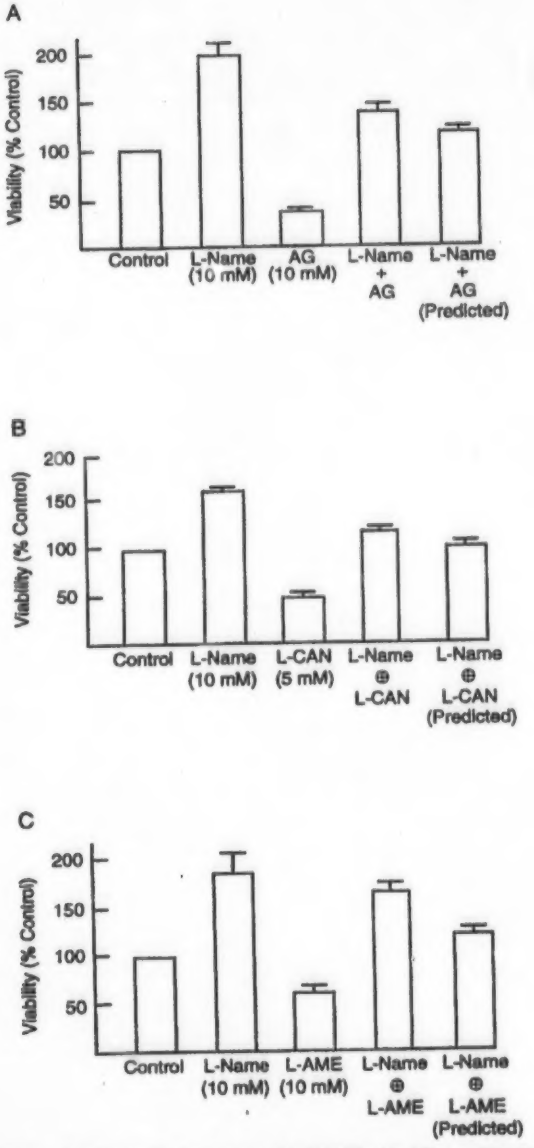

FIG. 8. Effect of coadministration of L-NAME with AG, L-canavanine, and L-AME on the toxicity of HD in neuron culture. Eight-day-old cultures were treated for 1 h with a combination of 10 mM L-NAME and AG (A), L-canavanine (B), or L-AME (C) prior to HD exposure and assayed for viability 24 h later. The concentrations of AG, L-canavanine, and L-AME were planned so that if they were exerting their activity at the same site as L-NAME, their additive activity should approximately cancel each other out. Data for the modulatory effects of the individual compounds on HD toxicity are shown, as well as the effect of coadministration (L-NAME + potentiating drug) on the toxicity of HD. Values represent the mean ± standard deviation of at least three separate experiments and are normalized against the LC50 of vehicle-pretreated HD-treated control cultures.

respect to the NOS pathway (Morgan, 1994). Spermidine and spermine were used as pretreatments in HD-treated cultures and were found to be toxic at 1.0 mM concentrations. At 100 μM, neither compound modulated the toxicity of HD, showing that polyamine metabolism is not likely to be involved in the toxicity of this CW agent.

The possible importance of the urea cycle to HD toxicity was investigated by testing four of its intermediates, arginine, ornithine, citrulline, and argininosuccinic acid (Fig. 1). Ornithine and argininosuccinic acid were inactive, while L-arginine and L-citrulline exhibited activity only at very high concentrations (25 and 100 mM, respectively). At these concentrations it is improbable that these compounds were conferring protection against HD toxicity by specifically perturbing the urea cycle. Rather, it is more likely that their protective effects were due to a remote similarity to an optimum (arginine-related) structure that somehow confers protection against HD. Similarly, inhibitors of arginase, which converts L-arginine to L-ornithine and urea (Fig. 1), were ineffective. Both AIAH and AIAP are potent inhibitors of arginase and when used as pretreatments were toxic at 100 μM concentrations. At nontoxic concentrations (10 μM) AIAH was inactive, while AIAP minimally increased the toxicity of HD. Once again, this increase in toxicity is probably due to an increased susceptibility of the cells to near-toxic concentrations of AIAP, rather than being a specific biochemical effect, i.e., arginase inhibition. This is further supported by the fact that both L-ornithine and L-lysine have also been shown to be effective inhibitors of arginase at low micromolar concentrations (Hrabak *et al.*, 1994; Robertson *et al.*, 1993) and exhibited no activity in these studies.

Several drugs tested in these studies (D/L-Arg, D/L-NAME) have been shown to be effective scavengers of hydroxyl free radicals at high micromolar concentrations (Dikshit *et al.*, 1996; Rehman *et al.*, 1997) and it is conceivable that the protective efficacy of these compounds against HD is due to the elimination of HD-induced free radicals. However, both the timing and the persistence of L-NAME protection mitigate against this explanation. In this study, as well as in previous studies (Sawyer, 1998; Sawyer *et al.*, 1996) L-NAME was found to be equally protective whether it was used as a pretreatment or when added to the cultures up to 3 h post-HD exposure. If HD were causing elevated hydroxyl radical levels, one would expect their production to be initiated fairly rapidly after HD exposure, with the protective efficacy of L-NAME being maximal at early time points and then falling off rapidly, i.e., not 3 h post-HD. More importantly, the reversibility of L-NAME protection also contradicts the role of hydroxyl radical scavenging. If L-NAME is removed from the cultures at any time point before or after HD exposure, the protective effects against HD toxicity are rapidly lost (Sawyer, 1998). If L-NAME was indeed scavenging hydroxyl radicals, then protection should be, to a large extent, irreversible.

A very surprising and puzzling aspect of these studies was the identification of several arginine-related compounds that demonstrated a pronounced, concentration-related, and reproducible potentiation of HD toxicity. These included the NOS inhibitors L-canavanine (Iyengar *et al.*, 1987; Teale and Atkin-

son, 1994; Umans and Samsel, 1992), MeG and AG (Griffiths *et al.*, 1993; Hasan *et al.*, 1993; Misko *et al.*, 1993), as well as guanidine and L-AME. That these compounds should be so closely structurally related to the protective drugs identified in this study seemed to be a remarkable coincidence and efforts were initiated to assess whether both sets of compounds were acting at the same site. Since the above studies did not shed any light on the mechanism of action of HD toxicity or arginine analogue effects, a direct mechanistic assessment of these compounds was not possible. In lieu of this approach, however, characterization of the effects of the drugs in question should supply an indirect indication as to whether they were exerting their effects at the same locus.

The protective effects of L-NAME have been extensively characterized previously (Sawyer, 1998; Sawyer *et al.*, 1996) and have been found to be unique. While L-NAME is an extremely effective antidote to HD in mature neuron cultures, it exhibits no efficacy in immature cells. Furthermore, the protection conferred by L-NAME in mature cultures is therapeutic in nature; with similar protective ratios being obtained against HD whether the cultures are treated 1 hour prior to HD exposure, or up to 3 h afterward. Three of the most effective compounds that exacerbated HD toxicity were compared to L-NAME. Although L-canavanine, AG, and L-AME were most effective in increasing the toxicity of HD in mature cultures, they did not alter its toxicity in immature, 1-day-old cells. Furthermore, these compounds were just as effective at increasing the toxicity of HD whether they were administered 1 h before or 2 h after the cultures were treated with HD. These results clearly showed that these compounds modulated the effects of HD in a fashion that was very similar to that of L-NAME, suggesting that they were exerting their effects at the same site. If this was indeed true, than their combined effects should negate each other. Experiments were therefore carried out in which mature cultures were pretreated with a combination of L-NAME and L-canavanine, AG, or L-AME. The concentrations of these drugs were predetermined so that their effects on HD toxicity should roughly cancel each other out—if their effects were directed toward the same site. For example, a concentration of L-NAME which increased the LC50 of HD by 200% (Table 1a) combined with a concentration of compound which decreased the LC50 by 200% (Fig. 5) should result in a drug combination with no modulatory activity on HD toxicity. Coadministration of L-AME with L-NAME lowered the protective effects of the latter compound, but not to the extent expected if the two compounds were acting at the same site. However, the combination of these two compounds produced a colored product which altered the results obtained with alamarBlue; visible inspection of the cultures showed that these two compounds did in fact neutralize each other's effects. The results with L-canavanine and AG were definitive; coadministration with L-NAME neutralized the modulatory action of the drug combination on the toxicity of HD. These results indicate that there is a strong possibility that the compounds that potentiate HD toxicity are acting at the same site as those drugs that protect against HD toxicity.

In summary, this study has identified eight additional compounds that protect against HD toxicity, as well as five that potentiate its toxic effects. Although how they exert their modulatory action on HD toxicity is not known, previous studies have shown that it is not through the inhibition of NOS, by the scavenging of toxic free radicals, or through a chemical interaction between HD and the drug. In addition, the current study also shows that their effects are not likely to be due to perturbations of flavoprotein or iron metabolism or to be associated with disturbances of the urea cycle or polyamine synthesis. These compounds are all structurally related to arginine and apparently exert their modulatory effects at a common site. Although the mechanism of action of these drugs was not identified, quantitative structure–activity relationship analysis of these compounds and their effects should assist in elucidating how they exert their modulatory effects on HD toxicity and possibly on the mechanism of action of HD itself.

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